EXPERIMENTAL STUDY

Effect of angiotensin ii type 1 (at1) receptor antagonist on the endothelial dysfunction in spontaneously hypertensive rats in correlation with the nitric oxide system

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Abstract

Background: **Hypertension is associated with impaired endothelial function, which can be explained by a decrease in nitric oxide (NO) generation or by an enhanced inactivation of NO after its release from endothelial cells.**
Objectives: **The aim of this study was to investigate the effect of long-term treatment with losartan, an angiotensin II (AT1) receptor antagonist, on endothelial dysfunction in an animal model of hypertension in relation to the nitric oxide system.**
Methods: **Losartan was administered to 48 sixteen-week-old spontaneously hypertensive rats, in a dose of 10 mg/kg bw/daily in drinking water, for 12 weeks. Systolic blood pressure (SBP) was measured at the beginning, after 4, 8 and 12 weeks of treatment, by the tail-cuff plethysmographic method. At each mentioned time point, a group of 12 animals was sacrificed and blood was withdrawn from the abdominal aorta. Plasma samples were used for determination of total nitrate/nitirite levels, cyclic guanosine monophosphate (cGMP) and endothelin (ET) 1 levels. Statistical evaluation of the results was performed by the use of a computer statistical programme Statistica for Windows 5.0.**
Results: **Losartan produced a significant decrease of SBP at all time points. On the other hand, long-term treatment with this AT1 receptor antagonist produced a significant increase of nitrate/nitrite and cGMP plasma levels. When we compared the values of SBP with plasma nitrate/nitrite as well as with cGMP values, a statistically significant correlation was established. A statistically significant decrease in plasma endothelin 1 values was found during the whole study period. Also, a positive correlation between SBP and plasma endothelin 1 concentrations was observed.**
Conclusions: **Long-term losartan (AT1 receptor antagonist) treatment, apart from its blood pressure lowering effect in hypertension, has beneficial effects on the endothelial dysfunction which is at least partially due to the activation of the nitric oxide system.** ***(Tab. 1, Fig. 2, Ref. 33.)***
Key words: losartan, hypertension, endothelial dysfunction, nitric oxide.

Vascular endothelial cells play a key role in cardiovascular regulation by producing a number of potent vasoactive agents, including the vasodilator molecule nitric oxide (NO) and the vasoconstrictor peptide endothelin (ET)-1. A dysfunction of the vascular endothelium has been implicated in the pathophysiology of a number of cardiovascular diseases, important among which is essential hypertension (1, 2). Impairment of NO synthesis, or increased inactivation of NO by superoxide radicals, may account for the increased peripheral vascular tone associated with hypertension, and can contribute to the clinical consequences of this condition (3, 4). Similarly, increased ET-1 synthesis, or increased smooth muscle sensitivity to ET-1, could account for the increased peripheral vascular tone and vascular hypertrophy (5). It is still not clear whether endothelial dysfunction is a primary or a secondary feature of hypertension. Therefore, modulation of endothelial function is an attractive therapeutic option in the treatment of hypertension.

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Angiotensin II (AT1) receptor antagonists are a group of drugs that are used in the treatment of hypertension. Their mechanism of action involves the specific blockade of Ang II (AT1) receptors, which inhibits vasoconstriction and prevents vascular and cardiac hypertrophy (6, 7). Preliminary findings suggest that these drugs have a potential to restore impaired endothelial function, according to the effects on potentiation of acetylcholine-induced vasodilatation of femoral vessels and forearm vasculature in essential hypertension (8, 9). Similar results were obtained during long-term treatment with angiotensin converting enzyme (ACE) inhibitors and calcium antagonists in animal models of hypertension (10). Their beneficial effect on the vascular endothelium was connected with the potentiation of the actions of NO, inhibition of the ET-1 actions and antioxidant activity. This study was undertaken to assess whether long-term administration of losartan, an angiotensin II receptor antagonist, influences endothelial dysfunction in an experimental model of hypertension in relation to the nitric oxide system.

Material and methods

Experimental design

The study was performed in male spontaneously hypertensive rats (SHR) (N=48), weighing from 200 g to 300 g (16 weeks old), obtained from the Animal Facility of the Department of Preclinical and Clinical Pharmacology and Toxicology, Medical Faculty, Skopje. The genetic predisposition, spontaneity and progressive rise of the blood pressure, as well as the pathological changes which occur in this type of animal are in close analogy to human essential hypertension. Animals were kept 6 in cages, maintained under controlled light and temperature conditions, fed a normal rat chow and had a free access to tap water. Chronic treatment with losartan (2-n-butyl-4-chloro-5-hydroxy-methyl-1-[(2-(1h-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, potassium salt), obtained from the pharmaceutical company, Alkaloid AD, Skopje, was performed every day for 12 weeks. The drug was administered in drinking water at a dose of 10 mg/kg bw/daily. Baseline blood pressure values were determined at the beginning of the study by the tail-cuff plethysmographic method. Blood pressure was also measured after 4, 8 and 12 weeks of losartan treatment. For evaluation of the effect of losartan treatment on the plasma nitrate/nitrite levels, cGMP levels and endothelin 1 levels and their correlation with the systolic blood pressure, a group of 12 animals was sacrificed at the beginning of the experiment, as well as after 4, 8 and 12 weeks of treatment, when the study was finished. Before sacrificing, animals were anaesthetized with pentobarbital (50 mg/kg b.w.), the blood was withdrawn from the abdominal aorta (6–8 ml) and collected into tubes with EDTA. Plasma samples for analysis were stored at -700 °C.

Blood pressure measurement

Seven days before beginning of the experiment, rats were trained daily for measurement of SBP (systolic blood pressure) by the tail-cuff plethysmographic method (IITC, Life Science, California, USA). Each day the rats were put in the measurement cages and placed in a room at 28 °C for 2 hours. SBP measurement were performed for 2 consecutive days at the same time. Eight measurements were carried out each day in each rat, and the maximum and the minimum values were rejected. To validate the tail-cuff method for BP measurement, eight rats were implanted with femoral artery catheters and underwent direct BP measurement method. The mean value of direct SBP compared with the mean value of indirect measurement showed correlation of 90 %.

Plasma nitrate/nitrite (NOx) concentrations measurement

Plasma samples were ultrafiltered with 10 kDa Millipore filters (pre-rinsed with redistilled water) and centrifuged at 6000 g, for 180 minutes at +40 °C. An amount of 40 l of the filtrate was used for the assay. Determination of total nitrate/nitrite concentration was performed by the use of commercial kit obtained from ALEXIS Corporation, Switzerland (Cayman Chemical Nitrate/Nitrite Colorimetric Assay kit, 850-001K101). The principle of the assay includes a two-step process: conversion of nitrate to nitrite utilizing nitrate reductase and addition of Griess Reagents which convert nitrite into deep purple azo compound. The absorbance due to this azo chromophore that accurately determines nitrite concentrations, was measured photometrically on Wallac 1420 Victor 2 aparatus (using microplates with 96 wells) at absorbance wave length of 540 nm.

Plasma cGMP measurement

Extraction of cGMP from plasma was made by the following protocol:

After adding 6 % trichloroacetic acid to plasma samples to provide a 100 g/L dilution and centrifugation at 2000 g for 15 minutes at 40 °C, supernatant was washed 4 times with 5 volumes of water saturated diethyl ether. The upper layer was discarded after each wash. The aqueous extract remaining was dried under a stream of nitrogen at 600 °C. The dried extract was dissolved in 500 l of assay buffer (supplied in the kit) prior to analysis. Determination of cGMP concentrations in plasma was performed by the use of RIA kit from Amersham Biosciences - cGMP [^{125}I] assay system (code RPA 525), that utilizes a highly specific activity [^{125}I] 2'-0-succinyl-cGMP tyrosine ester tracer, together with a high specific and sensitive antiserum. Separation of the antibody bound from free fraction was achived with a second antibody Amerlex-M preparation, allowing a magnetic separation.

Plasma endothelin 1 measurement

Endothelin 1 in plasma was determined with a commercial kit supplied from Amersham Biosciences (code RPA 545-Biotrack endothelin -1,2-high sensitivity [^{125}I] assay system), that offers measurment of ET-1 in the range 0623-79.74 pg/tube using an overnight delayed addition protocol. Endothelin 1 was extracted from plasma by the use of a separation protocol involving Amprep 500 mg C2 columns (code RPN 1913). The dried samples at the end were reconstituted with 250 l of assay buffer and measured in duplicate.

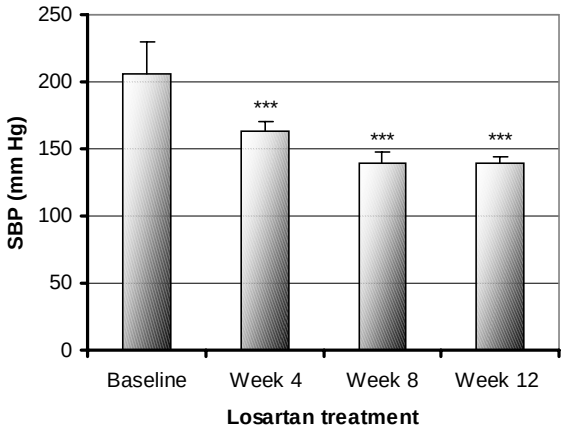

Fig. 1. The effect of chronic losartan treatment on the systolic blood pressure at different time points. SBP — systolic blood pressure (mmHg), *** p<0.001 vs baseline levels.

Statistical analysis

Statistical evaluation of the results was performed using the computer statistical programme Statistica for Windows 5.0. Results were expressed as means SD. Comparisons were made using the Student "t" test and one-way analysis of variance ANOVA. Correlations between systolic blood pressure (SBP) values and total nitrate/nitrite values, cGMP as well as endothelin 1 levels were expressed by Pearson coefficient of correlation (r). A p value <0.05 was considered to indicate a statistical significance.

Results

Our results show that chronic losartan treatment decreased SBP levels in SHR (Tab. 1, Fig. 1). The decrease was statistically significant after 4, 8 and 12 weeks of treatment in comparison to the baseline SBP levels ($p<0.001$). A significant decrease of the SBP ($p<0.001$) was also observed between the SBP values measured in week 8 compared to week 4. On the other hand no statistical significance ($p=0.852$) was found between the values of SBP determined in week 12 compared to week 8 (Tab. 1, Fig. 1).

Long-term losartan treatment produced a statistically significant increase in plasma nitrate/nitrite (NOx) levels ($p<0.001$) in all time intervals of the study in comparison to the control values (Tab. 1). We also found a significant increase between NOx levels measured after 8 weeks of treatment and NOx levels determined after 4 weeks of treatment ($p<0.001$), as well as between NOx levels measured after 12 weeks of treatment and NOx levels obtained after 8 weeks of treatment ($p<0.001$) (Tab. 1). When we compared the values of plasma nitrate/nitrites at all time points of the experiment with the SBP values, a statistically significant negative correlation was established ($r=-0.87$) (Fig. 2A).

Similar results to NOx were found with the cGMP plasma values. In our experiment losartan produced a statistically significant increase of plasma cGMP levels ($p<0.001$) during the all study periods comparing to the baseline levels (Tab. 1). A significant increase of cGMP was found between levels measured after 8 weeks of treatment and those determined after 4 weeks of treatment ($p<0.001$), as well as between cGMP levels measured after 12 weeks of treatment and the levels obtained after 8 weeks of treatment ($p<0.001$) (Tab.1). Comparing the cGMP values at all time points of the experiment with the SBP values, a statistically significant negative correlation was determined ($r=-0.86$) (Fig. 2B).

Twelve-week losartan treatment significantly decreased the values of endothelin (ET)1 in SHR ($p<0.001$) at all time points comparing to the control values measured at the beginning of the study (Tab. 1). There was also a significant decrease found between ET1 plasma levels determined after 8 weeks of treatment compared to those measured after 4 weeks of treatment ($p<0.001$), as well as between ET1 levels obtained after 12 weeks of treatment in comparison with the levels determined after 8 weeks of treatment ($p<0.001$) (Tab. 1). We also found a statistically significant positive correlation between SBP and ET-1 plasma levels ($r=0.79$) (Fig. 2C).

Discussion

Losartan potassium is the first long-acting and orally-active, nonpeptide angiotensin II (AT1) receptor antagonist, used in the management of hypertension. It binds competitively and selectively to the angiotensin II (AT1) receptor, thereby blocking Ang II-induced effects. In the present study, we have shown that long-term treatment with losartan lowers SBP in SHRs that represent a model of a human essential hypertension. Similar results were observed in several experiments where losartan was chronically administered to laboratory animals (11, 12, 13). Timmermans et al (14) suggested that the mechanism of action of losartan involves a blockade of Ang II induced vasoconstriction, blockade

Tab. 1. The effect of chronic treatment with losartan on the systolic blood pressure, plasma concentrations of nitrate/nitrite, plasma cyclic guanosine monophosphate and endothelin 1 levels (means SD) in spontaneously hypertensive rats.

	Baseline	Week 4	Week 8	Week 12
SBP (mmHg)	205.83 ± 24.48	162.67 ± 7.68***	139.58 ± 8.43***	139.25 ± 4.93*
Plasma NOx (μmol/l)	15.73 ± 2.2	237.277 ± 2.08***d	44.22 ± 3.42***e	45.56 ± 3.5**
cGMP (pg/ml)	1.05 ± 0.15	2.37 ± 0.22***g	2.98 ± 0.22***h	3.32 ± 0.25***i
Endothelin 1 (pg/ml)	3.6 ± 0.4	2.62 ± 0.26***j	2.33 ± 0.17***k	2.14 ± 0.16***l

SBP — systolic blood pressure, NOx — nitrate/nitrite, cGMP — cyclic guanosine monophosphate, *** p<0.001 vs baseline levels; a vs b, p<0.001; b vs c, p NS; d vs e, p<0.001; e vs f, p<0.001; g vs h, p<0.001; h vs i, p<0.001; j vs k, p<0.001; k vs l, p<0.001.

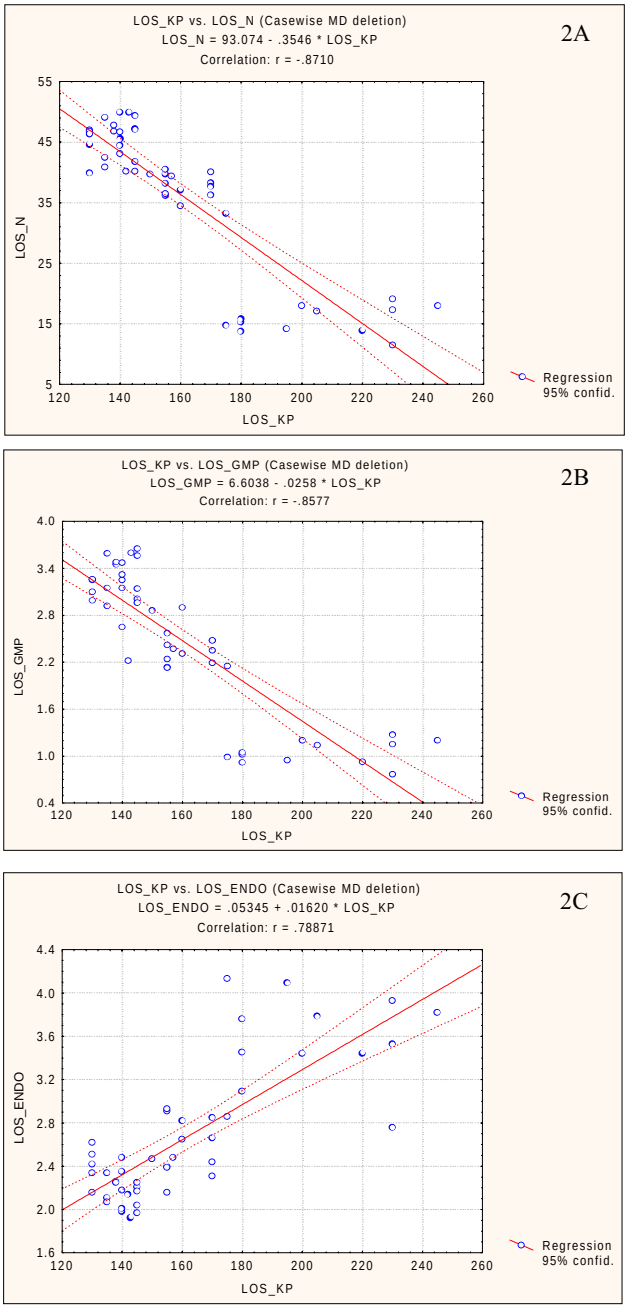

Fig. 2. Correlation between systolic blood pressure and: plasma nitrate/nitrite levels (Fig. 2A), cGMP levels (Fig. 2B) and endothelin 1 levels (Fig. 2C) during 12 weeks treatment with losartan.

of sympathetic function, an increased baroreflex sensitivity as well as complex effects to modify vascular hypertrophy and reactivity. On the other hand, Searles and Harrison (15) have reported that Ang II directly stimulates NO synthase activity in endothelial cells and that the released NO increases cGMP production in smooth muscle cells.

In our study, we found a significant increase in plasma nitrate/nitrite levels, as final products of NO synthesis, even after 4 weeks of treatment with losartan. They continued to increase markedly till the end of the experimental period. There was also a strong negative correlation between the rise of nitrates/nitrites and the decrease in the SBP. Our results were comparable with the results that have been previously reported in experimental animals and humans, indicating the finding that the antihypertensive effect of AT1 receptor antagonists was correlated with the nitric oxide system (16, 17, 18). It is well known that nitric oxide is synthesised from L-arginine by a family of nitric oxide synthases (NOS). NO exerts its effect by increasing intracellular cyclic guanosine monophosphate (cGMP) levels, which in turn lower intracellular calcium levels. In plasma, nitric oxide is oxidized to nitrite, which is stable for several hours. In whole blood, however, nitrite is rapidly converted to nitrate. Basally the produced NO is important in the regulation of vascular tone and blood pressure, as well as in the inhibition of platelet aggregation and platelet and monocyte adhesion to the endothelium. It has been demonstrated that deficiency of endogenous NO leads to impaired vasodilatation and a rise in peripheral vascular resistance, resulting in hypertension (19, 20, 21). On the other hand, it has been postulated that Ang II is responsible for the production of NO (22). The mechanisms leading to this are still controversial. Recent work suggests that simultaneously to the AT1 receptor blockade during the treatment with AT1 receptor antagonist, a stimulation of AT2 receptors by Ang II, occurs, and leads to activation of the kinin-kallikrein system and bradykinin release. Bradykinin then binds to its receptor on adjacent endothelial cells, causing the release of NO and stimulation of cGMP (23, 24).

In addition to the blood pressure lowering action, long-term losartan treatment in our study increased plasma cGMP levels, in the same manner as the plasma nitrates/nitrites. A number of studies have suggested this effect of losartan on vascular cGMP level (25, 26, 27). This effect of chronic AT1 blockade on aortic cGMP content was even more pronounced than that produced by equally antihypertensive chronic ACE treatment (28). The link between Ang II and the NO system was proven in bovine endothelial cells, where Ang II dose-dependently stimulated cGMP production (29).

Simultaneously with the lowering of blood pressure, we observed a significant endothelin 1 decrease in the plasma of treated animals, at all time points of the study. This finding implicates ET-1 in a role in hypertension. ET-1 is known as a member of the endothelin system that is present in the greatest abundance in the circulation. Different reports have shown plasma ET-1 to be slightly increased or normal in different rat models of hypertension and in hypertensive humans (30,31). However, because ET-1 release is in part Ang II dependent (32), AT1 receptor blockade might have the potential to reduce plasma ET-1 levels in conditions with renin-angiotensin activation. An interesting observation was published by Schiffrin et al (33) who wrote that ET-1 and ET-3, through their interaction with ETb receptors present in endothelial cells, may stimulate the release of nitric oxide and prostacyclin, which relax the underlying vascular smooth muscle. However, other studies have provided opposite results (similar to those observed in our study) indicating that endothelin indeed releases less endothelium-derived relaxing factor in SHR blood vessels. It has also been

demonstrated that Ang II may stimulate the production of endothelin in SHR (33). Thus, the exact significance of this potentially important mechanism remains to be elucidated.

We can conclude that simultaneously with its blood pressure lowering action, losartan improves endothelial dysfunction in a rat model of essential hypertension through the nitric oxide system. This indicates that AT1 receptor antagonists besides their antihypertensive effect, could be important therapeutic tools to prevent or reduce the development of endothelial dysfunction.

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